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Environmental hormones and their impacts on sex differentiation in fathead minnows

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ABSTRACT

Runoff from lands fertilized with animal manure from concentrated animal feeding operations (CAFOs) is a source of hormones to surface water. In this study we tested the hypothesis that larval fathead minnows exposed to sex steroids singly or in a "typical" CAFO mixture during sex differentiation would respond with changes in the expression of a set of target genes, leading to gonadal abnormalities later in life. In the first experiment, a static daily-renewal system was used to expose larvae during the period of 10–20 days post-hatch (dph) to either 5 ng/L 17β-trenbolone (17β-TRB) or 5 ng/L 17α-ethinylestradiol (EE_2). In a second experiment, fish were exposed from 0 to 45 dph in a flow-through system to a CAFO mixture composed of steroids and degradates (2–16 ng/L), atrazine and degradates (15–250 ng/L), and nitrate (3–11 mg/L). In the single hormone experiment, expression of genes involved in steroidogenesis (*cyp19a, cyp17*, and *star*) was decreased in females. In contrast, no differences in gene expression were observed in fish exposed to the CAFO mixture. However, the majority (84%) of treated males had testes containing an ovarian cavity, indicative of feminization, compared to 0% in the control males. Overall, our results show that: (1) changes in gene expression after single hormone exposures are sex-specific, with females more responsive than males; and (2) phenotypic alterations in testicular development can be elicited by a simulated "CAFO" mixture when fathead minnows are exposed during the first 45 days of development. More research is needed to further discern the complex response of fish to steroid mixtures, especially those associated with runoff from land-applied CAFO waste.

1. Introduction

Natural and synthetic hormones have been detected in aquatic environments. Important sources of hormones to surface waters are runoff from land on which animal manure has been applied as a fertilizer and discharge of treated municipal wastewater (Gall et al., 2011). Concentrated animal feeding operations (CAFOs) produce large amounts of animal waste that contain nutrients, as well as natural and synthetic hormones (Burkholder et al., 2007). In addition, CAFOs are often associated with corn fields that commonly apply herbicides during late spring-early summer (April–June), coinciding with fish spawning (Stoeckel et al., 2012). One herbicide of interest is atrazine, which is widely used and reported as an endocrine disruptor (Weber et al., 2013). Midwest agricultural fields have subsurface tile-drain networks that facilitate transport of excess water from agricultural fields to a ditch network system. As a result, excess nutrients, herbicides, and hormones can be quickly mobilized into ditches (Gall et al., 2011), ultimately reaching streams and rivers.

Effects of sex steroids on fish and wildlife reproduction have been widely studied (Orberdorster and Cheek, 2000; Tyler et al.,

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1998). However, there is significantly less known about the effects of androgens compared to estrogens (Ankley et al., 2003; Jensen et al., 2006), and almost no studies are available that have tested androgen and estrogen hormones and degradates in a mixture. Using information on the estrogenic potency (indexed to 17α-ethinylestradiol, EE_2 = 1, maximum) and androgenic potency (indexed to 17β-trenbolone, 17β-TRB = 1 maximum) of the mixture (Thorpe et al., 2003) and assuming concentration addition (Brian et al., 2005; Silva et al., 2002; Zhang et al., 2009), predictions of biological effects of steroid mixtures could be determined. However, there has been little exploration into the testing of mixtures containing endocrine disrupting compounds with dissimilar modes of action. Furthermore, to our knowledge, no controlled studies have been conducted exposing fish larvae or juveniles to a mixture of hormones, pesticides, and nutrients similar to those found in the environmental matrices, such as CAFO runoff.

Using fathead minnows we tested the overarching hypothesis that larvae exposed to sex steroids singly or in a "typical" CAFO mixture while undergoing sex differentiation (10–45 days post hatch, dph) would respond with changes in the expression of genes involved in this process, leading to gonadal abnormalities later in life. We tested this hypothesis in two experiments: one that evaluated molecular responses after exposure to single synthetic sex steroids; and a second experiment that evaluated molecular and organ (gonad) responses after exposure to a CAFO mixture (containing sex steroids and degradates; atrazine and degradates; and nitrate). Specifically, we hypothesized that activation of genes critical to ovarian development (*cyp19a* and *esr1*) would be observed in fish exposed to EE_2 or an "estrogenic" mixture. Analogously, an activation of genes critical for testicular development (*dmrt1* and *ar*) was expected after exposure to 17β-TRB or an "androgenic" mixture. We also expected a skewed phenotypic sex ratio toward males in larva exposed to the CAFO mixture, based on our previous studies showing this response in fathead minnows exposed from 0 to 45 dph to ditch water from a CAFO site containing a similar chemical mixture (Leet et al., 2012).

2. Methods

2.1. Animal model and experimental design

Fathead minnows used for all experiments were spawned at the Purdue University Aquatic Research Laboratory from fish reared at USEPA's Mid-Continent Ecology Division Laboratory in Duluth, MN. These fish contain a sex-linked DNA marker used for genotyping gender (Olmstead et al., 2011).

In the first experiment, a static, daily-renewal system was used to expose fathead minnow larvae (10–20 dph) to either 5 ng/L (18 pM) 17β-TRB, a synthetic androgen or 5 ng/L (17 pM) EE_2, a synthetic estrogen. These treatments were chosen because they are environmentally relevant concentrations that are sublethal (Shved et al., 2008). Fathead minnows were exposed during this developmental period to target gonadal differentiation (van Aerle et al., 2002; Uguz, 2008). Exposures were conducted in 18 6-well plastic plates with mesh well inserts (Corning, Inc., NY, USA) to facilitate daily water changes. Embryos (<24 h post fertilization, hpf) were removed from breeding substrates, triple rinsed in reverse osmosis (R/O) water reconstituted to a hardness of 3 meq with Replenish® (Seachem, Inc., Madison, GA, USA), and individually placed in a well containing a 10 mL total volume of clean reconstituted R/O. Fish were reared in clean water during the first 10 dph and those larvae were (total N = 101) split into groups to either be used as (N = 30 larvae) or exposed to the synthetic hormones from 10 to 20 dph (EE_2 N = 35 larvae; 17β-TRB N = 36). Survival and abnormalities were recorded daily. At the end of the exposure, fish were euthanized with MS-222, measured (±0.1 mm), and processed for gene expression analyses and genotyping (controls N = 29; EE_2 N = 28; 17β-TRB N = 32). In order to match genetic and phenotypic sex, RNA was extracted from the midsection of each larva and gene expression data matched to genetic sex. This also allowed for the determination of sex-specific body measurements for these fish. Water samples were pooled from the exposed and control wells daily after water exchange for confirmatory chemical analysis.

The second experiment consisted of a flow-through 45 days exposure of fathead minnows to a contaminant mixture that mimicked chemicals detected at an Indiana CAFO field site (Gall et al., 2011; Leet et al., 2012). The mixture contained the following nominal hormone concentrations in ng/L: 20 estrone (E_1; 74 pM), 10 17α-estradiol (17α-E_2; 37 pM), 10 17β-estradiol (17β-E_2; 37 pM), 10 estriol (E_3; 35 pM), 30 testosterone (*T*; 104 pM), 10 androstenedione (AND; 35 pM), 10 17α-trenbolone (17α-TRB; 37 pM), and 10 17β-TRB (37 pM). In addition, 0.110 μg/L (510 pM) atrazine and 10 mg/L nitrate were included. The exposure system consisted of 24, 9.5-L glass tanks, half of which received the chemical mixture in R/O reconstituted water a multiposition, microelectric valve actuator system (Valco Instruments Co. Inc., Houston, TX). Control tanks were set-up in the same system only receiving R/O water. Each tank received a flow of 3 mL/min and continuous aeration through a small aerator stone. One clutch of eggs still attached to the breeding substrate was placed in each tank (control N = 12; exposed N = 12). All but 150 eggs were removed from the substrate after eggs eyed to ensure enough eggs hatched for determination of sex ratios at 45 dph, using a combination of gonadal histology and genotyping (n = 20–50 juveniles/tank). Juveniles were weighed (±0. 1 mg wet) and measured (±1 mm) at the end of the study. Fish (n = 20) from three control and three exposed tanks were chosen for examination of gene expression changes at 20 dph. During the exposure, water samples were periodically collected for chemical analysis from the outflow of random tanks using 1 L amber glass bottles.

For both experiments, embryos/juveniles were incubated in a temperature-controlled chamber (24 ± 1 °C) with a photoperiod of 16 L:8 D. Dissolved oxygen was maintained at a minimum of 6 mg/L. From 0 to 4 dph fish were fed diluted Rotifeast (Reed Mariculture, Inc., Campbell, CA, USA); *ad libitum* live brine shrimp larvae (Artemia, Brine Shrimp Direct, Ogden, UT, USA) three times daily (twice daily on weekends) from 5 to 20 dph; and adult brine shrimp twice daily from 21 to 45 dph. All Institutional Animal Care and Use Procedures were approved through the Purdue University Animal Care and Use Committee (protocol # 11-038).

2.2. Exposure solutions and water chemistry analysis

Hormones were dissolved in 1 mL methanol and diluted into 1 L sterile deionized water for stock solution preparation, then sonicated for 30 min and stirred (on ice) overnight to ensure complete dissolution. Working solutions were prepared with sterile R/O water. The final concentration of methanol was 0.0000005%, negating the need for a vehicle control. Hormones and pesticides were quantified using solid phase extraction, eluting with methanol, evaporating eluant, and reconstituted residues in methanol (0.5 mL) followed by high performance reverse-phase liquid chromatography–tandem electrospray ionization mass spectrometry (HPLC/ESI–MS/MS) for the hormones (details provided Supplemental Material of Gall et al., 2011) and GC/MS for the pesticides.

For the CAFO mixture study, water chemistry is presented as the average concentration of the different analytes during three developmental periods representing pre- (0–9 dph), early (10–20 dph) and late (21–45 dph) sex differentiation for the fathead

minnow (Uguz, 2008). Water samples were taken throughout the experiment and a weighted average was calculated for each developmental period and adjusted for the number of days in each period for an estimation of exposure. The adjusted average concentration (C_{ave}) for each developmental period was calculated as follows:

$$C_{ave} = \left\{ \frac{((C_1 + C_2)/2) \times T_1 + ((C_2 + C_3)/2) \times T_2 + \cdots}{T_{dp}} \right\}, \quad (1)$$

$C_{1,2,3,\ldots}$ is the chemical concentration measured in water collected from the flow-through system. $T_{1,2,\ldots}$ is the duration (number of days) between water sample collections. T_{dp} is the total number of days the average concentrations is being adjusted for in a particular developmental period (*i.e.*, 0–9, 10–20, or 21–45 dph).

2.3. Genotyping and gonadal histology

Genetic sex was determined at 20 dph in fish from the single hormone exposure experiment and at the end of the 45 days CAFO mixture study using published methods (Leet et al., 2013; Olmstead et al., 2011). Although larvae were also sampled for gene expression analysis at 20 dph during the 45 days CAFO mixture experiment, the preservation of these samples in RNAlater (Sigma–Aldrich Corp., St. Louis, MO, USA) interfered with the polymerase chain reaction (PCR) for genetic identification of sex so genotyping was not possible for those samples. Genetic sex was compared to gonadal histology for 45 dph juveniles from the CAFO mixture study. Six replicate tanks (=3 for control and treated) were chosen for histological analysis (20–50 fish/tank, 240 fish total). Gonadal histology was not conducted in the 20 dph larvae for either exposure experiment since gonads are too immature to evaluate histologically at this age. Gene expression analysis for 20 dph larvae was conducted on 20 larvae from three control tanks and three treated tanks in the CAFO mixture study, of which one treated and one control tank were the same as those examined for histological sex at 45 dph.

Midsection samples collected at 45 dph were placed in histology cassettes and fixed in Davison's fixative (USEPA, 2006) for 24 h. Samples were processed by the histology laboratory at the Purdue University Animal Disease Diagnostic Laboratory, using standard hematoxylin and eosin staining of 5 μm thick sections. At least three sections were examined for each fish and blindly evaluated by two individuals using Uguz (2008) as a guide.

2.4. Gene expression

Gene expression was conducted on 20 dph larvae from both experiments. Seven genes were chosen for quantitative PCR (qPCR) analysis using previously described methods (Leet et al., 2013). Double sex and mab-3 related gene 1 (*dmrt1*; F: 5′-AGGTCGTGGGTGATGTGAAT-3, R: 5′-GGCCACTGCAGAGCTTAGAG-3′) is a transcription factor that is involved in sex determination. Steroidogenic acute regulatory protein (*star*; F: 5′-TGTCCGCTGTGCCAAAC-3′, R: 5′-GCTCTTACAAATCCTTTCTTCTC-3′) is a cholesterol transport protein involved in the production of steroid hormones. Cytochrome P450 19 gonad isoform (*cyp19a*; F: 5′-CAGGAGTTACAGGATGCCATCA-3′, R: 5′-CCGACCAGCTAAAACAGTTTCC) and cytochrome P450 17 (*cyp17*; F: 5′-AAAGGAAGATGAATGGATTTG-3′; R: 5′-GCAGTTGTTGAAAGAAGATG-3′) are steroidogenic enzymes. Estrogen receptor isoform 1 (*esr1*; F: 5′-CACCCACCAGCCCTCAG-3′; R: 5′-CACCTCACACAGACCAACAC-3′) and androgen receptor (*ar*; F: 5′-ATGGGAGTGATGGTCTTTG-3′; R: 5′-AGGTCTGGAGCGAAATAC-3′) are intracellular nuclear transcription factors involved in steroid signaling pathways. Ribosomal protein L8, *rpl8* (F: 5′-CTCCGTCTTCAAAGCCCATGT-3′; R: 5′-TCCTTCACGATCCCCTTGATG-3′), was used for housekeeping because it has been shown to be a stable gene for fathead minnow exposure experiments (Filby et al., 2007). No differences in *rpl8* expression were observed between genders or treatments (data not shown). All primer efficiencies were between 92 and 110%. Gene expression analysis was done on fish samples from the static single hormone exposure study that could be definitively sexed by genotyping, and for which enough total RNA was extracted to measure all seven genes (controls: =8 males, 11 females; EE_2: =12 males, 12 females; 17β-TRB =12 males, 11 females). All samples were flash-frozen in liquid nitrogen, except those collected at 20 dph from the CAFO mixture flow-through exposure which were preserved in RNAlater. The preservation of samples in RNAlater caused interference in the genotyping PCR for identifying sex, so it was not possible to genotype these samples. Expression of *cyp19a* has been shown to be sexually dimorphic in 20 dph fathead minnows (Leet et al., 2013), so its expression was used to define presumptive gender in the CAFO mixture exposure where genetic sex could not be identified (controls: 3 tank replicates, =32 males, 28 females; exposed: 3 tank replicates, =39 males and 21 females). A ΔCt value of <12 was considered female and >13.5 was considered male because there was differential expression with no ΔCt values between 12 and 13.5. Samples were used if enough RNA was extracted from individual fish to analyze all seven genes. Mean cycle threshold (Ct) values were calculated for the target and reference genes. Expression of target genes was normalized relative to the expression of *rpl8* ($\Delta Ct = Ct_{targe\,gene} - Ct_{rpl8}$). The normalized expression of the target genes was quantified by determining the $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008).

2.5. Statistical analyses

Statistical analyses were performed with JMP 8. An ANOVA was performed to test for significant differences between treatment groups within each sex for the static renewal exposure. A student *t*-test was used to determine significant differences between control and exposed group within each sex for the flow-through exposure. Significance was declared at $p < 0.05$. All data are presented as sex-specific mean ± SE. Sex ratios were compared across treatments using a chi-square test.

3. Results

3.1. Single hormone studies

Measured concentrations of EE_2 and 17β-TRB were 3.3 ± 0.4 ng/L and 3.3 ± 0.6 ng/L, respectively. Neither chemical was detected in the control water samples. Hatch rate for all embryos was 94%, and during the 10 day exposure period (10–20 dph) survival was 97% for controls, 80% for EE_2, and 89% for 17β-TRB (Table A.1). Although EE_2-exposed fish were significantly larger than those treated with 17β-TRB, neither group was significantly different to controls (Table A.1).

The genotypic sex of all fish sampled was 50% male. Sex-specific gene expression responses are shown in Fig. 1, by gender. In females, *dmrt1* was upregulated by EE_2 and downregulated by 17β-TRB. Also, females exposed to either chemical exhibited significant downregulation of *cyp19a*, *cyp17* and *star* expression. The only significant change in males exposed to 17β-TRB was a downregulation in the expression of *star*. Upregulation of *esr1*was observed in both males and females exposed to EE_2. No significant changes in *ar* expression were observed in any treatment or sex.

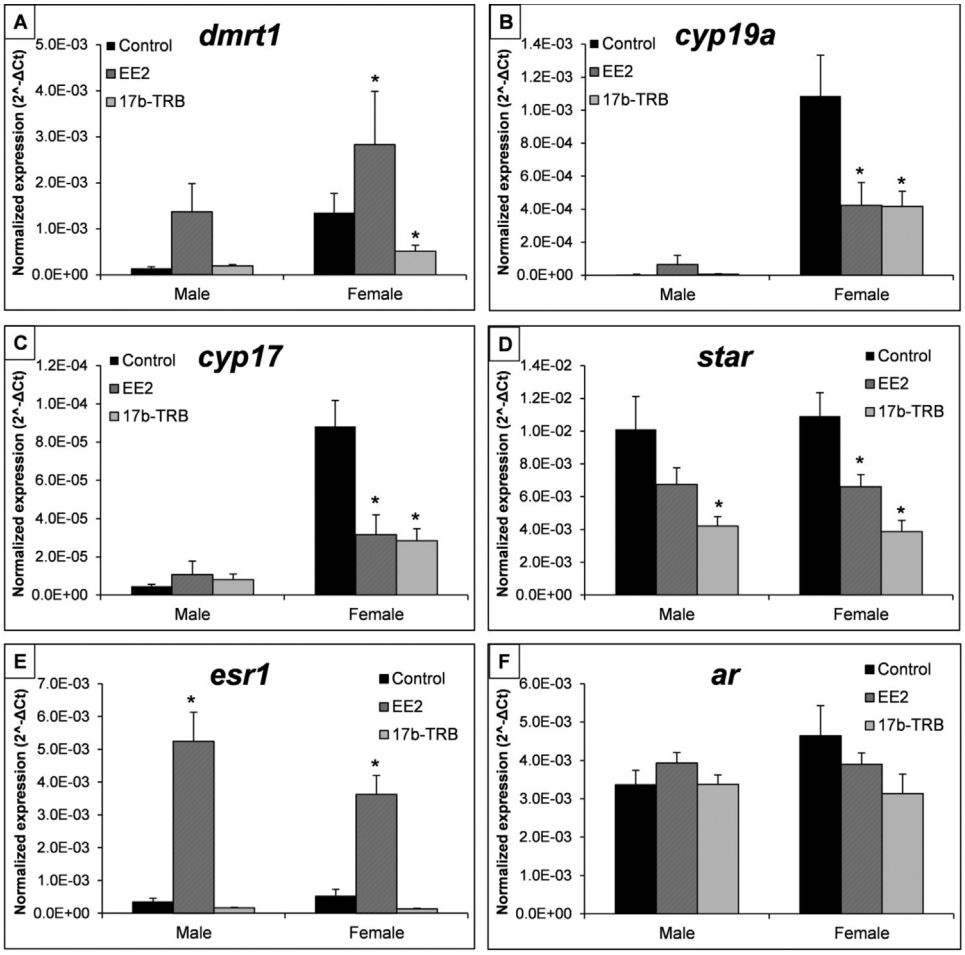

Fig. 1. Summary of gene expression changes in 20 dph fathead minnow larvae after exposure to 3.3 ng/L EE_2 or 3.3 ng/L 17β-TRB for 10 days. Gene expression for (A) double sex and mab-3 related gene 1 *dmrt1*, (B) cytochrome P450 19, *cyp19a*, (C) cytochrome P450 17, *cyp17*, (D) steroidogenic acute regulatory protein, *star*, (E) estrogen receptor 1, *esr1*, and (F) androgen receptor, *ar*, was normalized to *rpl8* (ΔCt method). Error is indicated by the bars.

Table 1
Summary of average chemical concentrations from three replicate tanks for the 45 days CAFO mixture exposure.

	Equivalancy factor	E01			E03			E04		
		0–9 dph	10–20 dph	21–45 dph	0–9 dph	10–20 dph	21–45 dph	0–9 dph	10–20 dph	21–45 dph
Estrone	0.28	5.34	4.96	4.59	4.93	4.90	4.24	5.34	2.25	6.54
17α-Estradiol[a]		2.88	4.38	5.19	5.66	5.07	4.54	4.73	2.51	6.59
17β-Estradiol	1.00	1.91	3.00	3.15	3.85	3.24	3.99	2.38	1.36	5.84
Estriol	0.05	3.53	6.58	9.58	5.94	7.00	8.51	6.10	3.35	11.92
Sum of estrogen equivalents[b]		3.58	4.72	4.91	5.53	4.97	5.61	4.18	2.16	8.27
Testosterone	0.078	2.19	2.14	0.74	1.75	1.68	0.56	1.89	0.45	0.08
Androstenedione	0.091	17.91	14.05	11.44	10.15	9.31	10.83	17.58	7.93	13.41
17α-Trenbolone	1.00	7.67	9.55	6.62	5.15	5.53	7.08	6.30	2.31	8.59
17 β-Trenbolone	0.47	4.72	6.73	3.10	3.43	3.20	3.67	4.21	1.49	5.42
Sum of androgen equivalents[b]		11.69	14.16	9.17	7.82	8.01	9.83	10.02	3.77	12.37
Atrazine	1.00	84.08	153.88	164.26	42.71	110.65	60.77	58.53	35.82	157.30
Desethylatrazine	0.29	25.55	32.77	24.24	9.60	26.91	8.22	20.00	8.64	30.86
Deisopropylatrazine	0.21	43.50	54.82	12.48	17.70	66.73	13.92	35.00	13.18	52.52
Sum of atrazine equivalents[c]		100.66	174.94	173.99	49.22	132.47	66.09	71.70	41.10	177.32

[a] 17α-Estradiol was not included in the equivalency calculations because there is no published information on binding affinity for these hormones to fathead minnow ER.
[b] Total equivalents = adjusted hormone concentration (ng/L). Relative Binding Affinities (RBAs) presented as percent (RBA of 100% = 1.0) used as equivalency factors. Estrogen RBAs are relative to 17β-estradiol (Denny et al., 2005). Androgen RBAs are relative to 17α-Trenbolone (calculated using values from Wilson and Cardon, 2007).
[c] Atrazine equivalency factors for atrazine degradates were based on relative potency based on LC_{50} data (Ralston-Hooper et al., 2009).

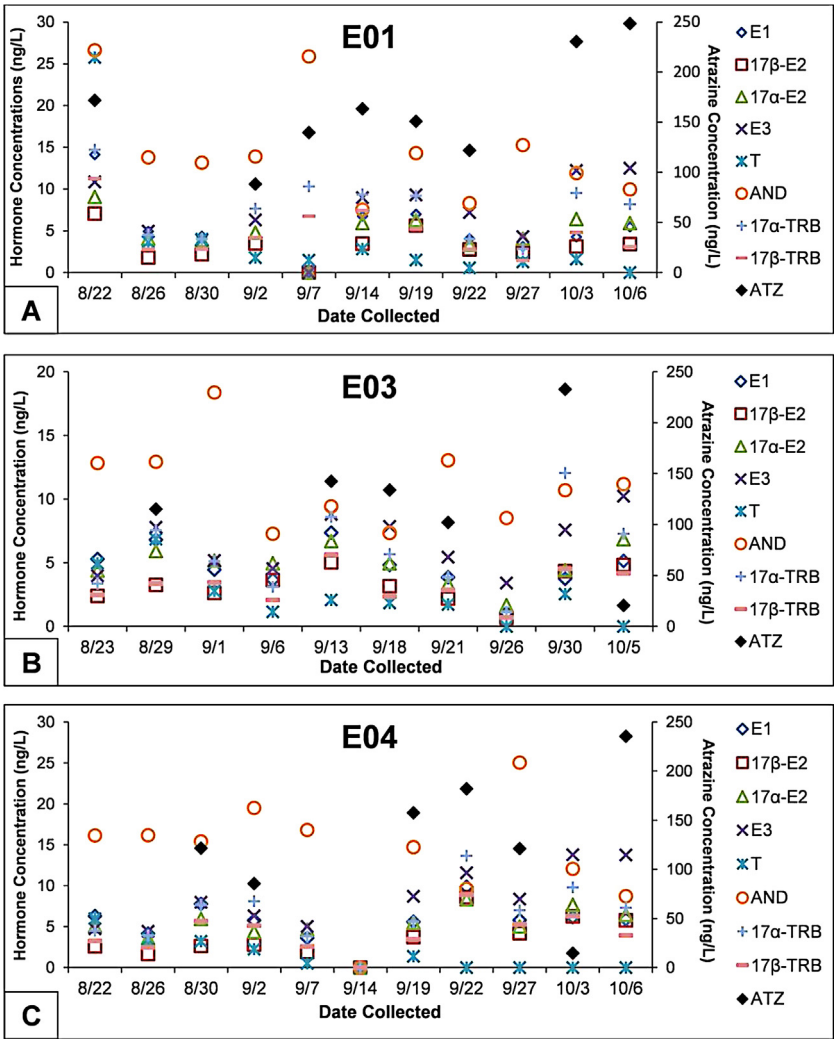

Fig. 2. Hormone and pesticide concentrations at various time points during the 45 days mixture flow-through study in three different tanks, (A) E01, (B) E03, and (C) E04. E_1, estrone; 17α-E_2, 17α-estradiol; 17β-E_2, 17β-estradiol; E_3, estriol; T, testosterone; and, androstenedione; 17α-TRB, 17α-trenbolone; 17β-TRB, 17β-trenbolone; ATZ, atrazine.

3.2. CAFO chemical mixture

Water chemistry data are summarized in Figs. 2 and A.1. With the exception of AND, all nominal chemical concentrations were below target concentrations. In control tanks, AND was also detected ranging from 7 to 16 ng/L (data not shown). The only other hormones detected in control tanks were E_1 and 17β-E_2, which ranged from 0.2 to 0.3 ng/L and from 1.2 to 1.3 ng/L, respectively. Atrazine in exposed tanks varied widely from 15 to 250 ng/L and nitrate ranged from 2.6 to 10.6 mg/L. An overall summary of average concentration values for each mixture tank from which gonadal histology was analyzed at 45 dph are presented in Table 1.

Sex genotyping was not possible in 20 dph fish, so high *cyp19a* expression was used to identifying presumptive females (Leet et al., 2013; see Section 2.4 for details). Sex-specific gene expression responses to the CAFO mixture are shown in Fig. 3. Regardless of gender, at 45 dph treated juveniles were larger compared to controls (Table 2). Genotypic sex ratio did not differ between controls (50 ± 6% males) and treated (56 ± 6% males) (Table 2). All genotypes matched the histological phenotypes except for two fish in control tank C05 (genotypic males identified phenotypically as females), one fish in experimental tank E01 (genotypic male identified phenotypically as female), and two fish in experimental tank E03 (genotypic females identified phenotypically as males). Of the males exposed to the CAFO mixture, 84% had ovarian cavities (Fig. 4); while no cellular abnormalities were observed in testes from control males (0% had ovarian cavities). These cavities varied in size and were formed because of the persistence of the cranial peritoneal attachment.

4. Discussion

In this study we (1) evaluated the use of expression changes in genes known to be involved in sex differentiation and gonadal development as indicators of molecular effects after exposure to potent sex hormones, and (2) determined if molecular changes under a more "natural" scenario, in which fish were exposed to a chemical mixture typical of a landscape dominated by CAFOs and corn fields, would result in gonadal phenotypic changes.

4.1. Single hormone studies

Larvae exposed to a nominal concentration of 5 ng/L EE_2 were larger than those exposed to 5 ng/L 17β-TRB, but neither was significantly different from controls. Ankley et al. (2003) reported

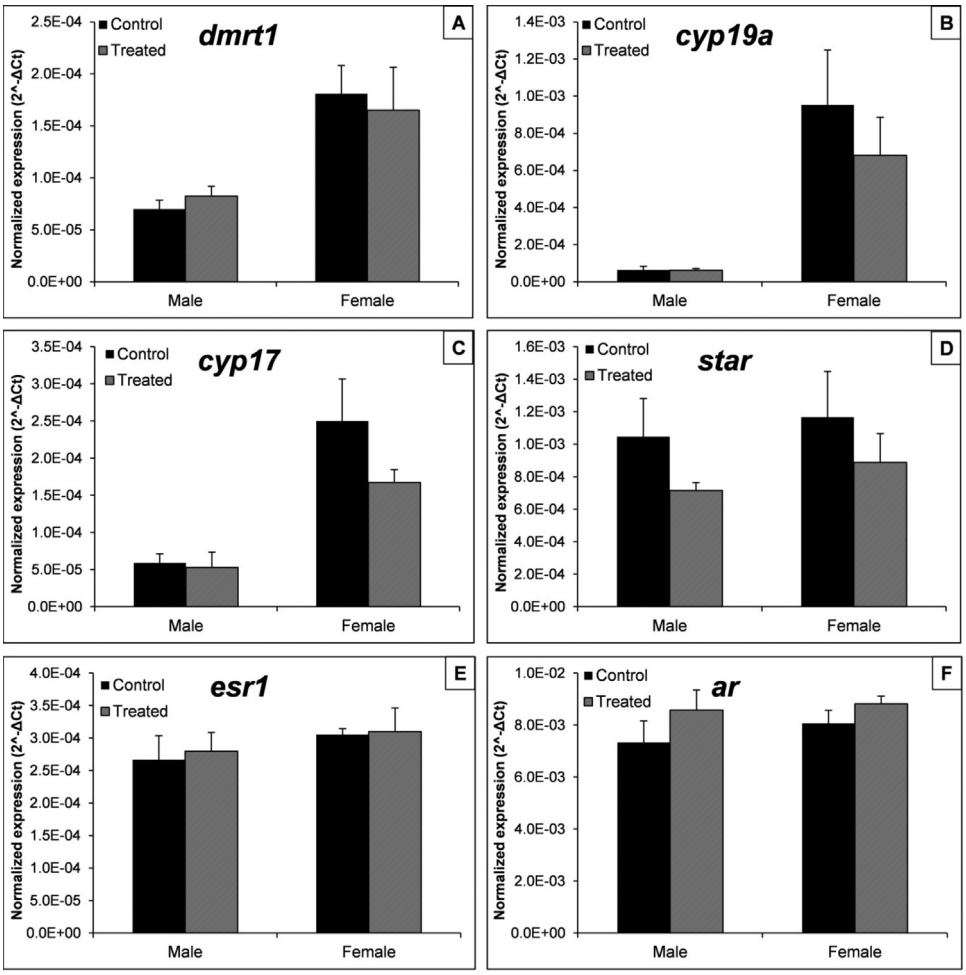

Fig. 3. Summary of gene expression changes in 20 dph fathead minnow larvae after exposure to CAFO chemical mixture. Gene expression for (A) double sex and mab-3 related gene 1 *dmrt1*, (B) cytochrome P450 19, *cyp19a*, (C) cytochrome P450 17, *cyp17*, (D) steroidogenic acute regulatory protein, *star*, (E) estrogen receptor 1, *esr1*, (F) androgen receptor, *ar*, was normalized to *rpl8* (ΔCt method). Significant differences from the control group within gender are indicated by an *(student *t*-test, $p \leq 0.05$).

Table 2
Mean ± SE of data measured in 45 days post hatch (dph) fathead minnow juveniles in the CAFO mixture study. Tank ID's underlined were used for genotyping and gonadal histology; bold indicates samples taken at 20 dph and used for gene expression.

Treatment	Tank ID	Survival[a] (%)	Overall body weight (mg)	Male body weight (mg)	Female body weight (mg)	Overall body length (mm)	Male body length (mm)	Female body length (mm)	Sex ratio (% males)
Control	C01	61	46.6 ± 3.0			17.1 ± 0.3			
	C02	41	102.8 ± 7.4	108.3 ± 14.3	98.9 ± 6.2	22.4 ± 0.4	22.5 ± 0.7	22.2 ± 0.4	50
	C04	74	46.3 ± 4.1			17.0 ± 0.5			
	C05	75	68.3 ± 4.5	62.1 ± 6.4	75.6 ± 11.3	18.7 ± 0.4	18.0 ± 0.5	19.6 ± 0.8	62
	C06	95	48.2 ± 4.0	49.0 ± 6.1	48.2 ± 6.2	17.3 ± 0.4	17.7 ± 0.8	17.1 ± 0.7	39
	C07	87	71.6 ± 4.1			20.0 ± 0.3			
	C08	62	134.3 ± 11.3			24.2 ± 0.7			
	C11	87	54.5 ± 4.0			18.1 ± 0.4			
	C12	59	70.4 ± 5.1			19.8 ± 0.5			
	Mean[b] ± SE	**71 ± 6**	**67.2 ± 2.1**	**70.1 ± 5.8**	**68.9 ± 5.15**	**19.0 ± 0.2**	**19.1 ± 0.4**	**19.2 ± 0.5**	**50 ± 6**
CAFO mixture	E01	54	104.9 ± 4.7	101.6 ± 6.0	109.5 ± 11.0	22.1 ± 0.3	21.8 ± 0.5	22.5 ± 0.6	54
	E02	81	85.5 ± 5.9			20.8 ± 0.5			
	E03	44	248.0 ± 15.9	249.6 ± 28.5	245.1 ± 22.3	28.6 ± 0.5	28.3 ± 1.1	28.7 ± 0.6	47
	E04	73	50.2 ± 3.4	54.5 ± 4.6	43.3 ± 4.7	17.6 ± 0.4	18.0 ± 0.5	16.9 ± 0.7	67
	E05	87	98.1 ± 16.4			20.8 ± 0.3			
	E06	75	91.9 ± 6.1			20.6 ± 0.4			
	E07	43	111.5 ± 6.1			21.3 ± 0.3			
	E08	99	60.3 ± 4.1			18.9 ± 0.4			
	E09	78	144.3 ± 9.7			24.5 ± 0.5			
	E10	93	55.6 ± 6.4			17.9 ± 0.5			
	E11	43	60.5 ± 5.7			18.3 ± 0.5			
	Mean ± SE	**69 ± 6**	**93.3 ± 3.1***	**96.1 ± 9.7***	**112.1 ± 14.9***	**20.6 ± 0.2***	**20.6 ± 0.6***	**21.4 ± 0.9****	**56 ± 6**

[a] Survival of hatched embryos throughout exposure duration (45 days).
[b] Bolded means are the average of tank means.
* Significant differences from controls (Student t-test, $p < 0.05$.).

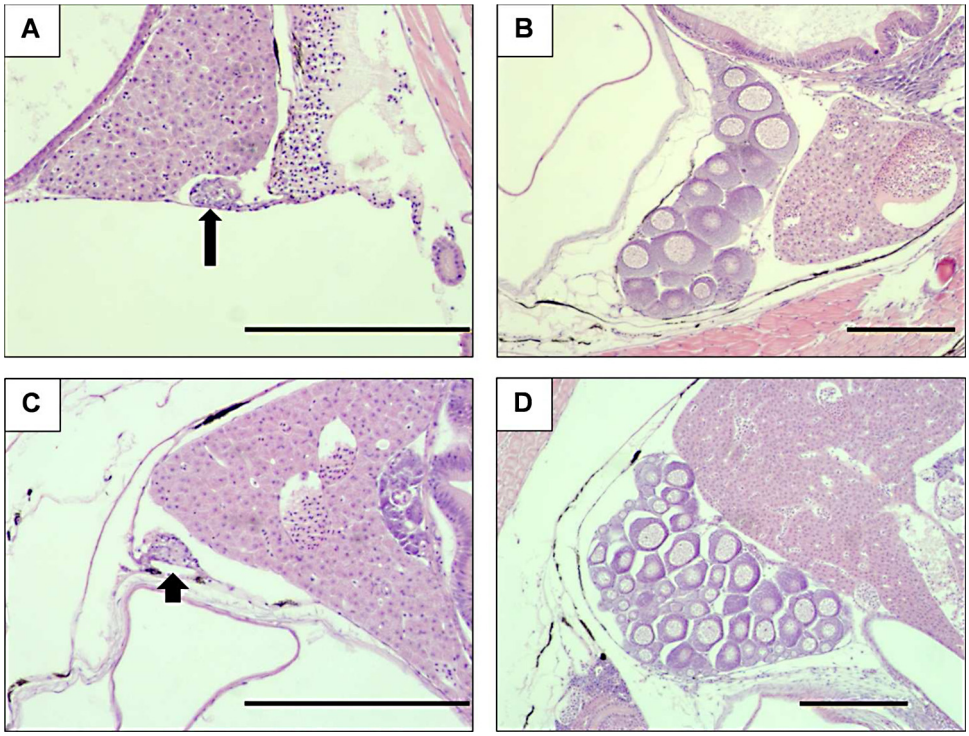

Fig. 4. Examples of gonads from the control group (A male, B female) and the experimental group (C male, D female) from the 45 days CAFO mixture flow-through exposure experiment. Arrow in *A* = testes tissue. Arrow in *C* = ovarian cavity in male testes tissue. Bar = 100 μm.

increased in adult female fathead minnow weights with 17β-TRB, but at concentrations well in excess of the ones tested in the current study. A decreased in body length has been reported in sheepshead minnows (*Cyprinodon variegatus*) exposed to much higher 17β-TRB (0.87 μg/L) for 42 weeks (Cripe et al., 2010). The lack of significant change in body length of hormone exposed larvae compared to the control in this study is consistent with that seen by Johns et al. (2011) in 7 days exposure of fathead minnow embryos to 2 ng/L EE_2. This indicates the lack of significant change in body length may be due to the early life-stage we were assessing and the relatively low concentration of hormones in the current experiment.

The gene expression data from the present study demonstrated that the period of gonad development we investigated is sensitive to exogenous hormones. Female fathead minnows appeared more sensitive to hormone exposure during early gonadal development than males. This is likely due to female gametogenesis beginning earlier in development than males (about 10 dph *vs* 25 dph, respectively; Uguz, 2008). Presumably steroidogenesis within the hypothalamic-pituitary-gonadal axis is more sensitive in females between 10 and 20 dph than males due to this earlier onset of gametogenesis.

We the exogenous hormones present are filling the role of endogenous hormones needed during development, therefore responding negatively on the feedback loop for steroidogenesis at this early stage in development (10–20 dph). We saw downregulation of *star* in females exposed to EE_2 and both sexes exposed to 17β-TRB. Sharpe et al. (2007) also observed a decrease in *star* expression after estrogen exposure in goldfish (*Carassius auratus*), as did Filby et al. (2006) in adult male fathead minnows. A similar response of downregulation of genes involved in steroidogenesis was also observed in Japanese medaka (*Oryzias latipes*) exposed to EE_2 (downregulation of *cyp19a* and *cyp17*) and 17β-TB (downregulation of *star*) (Zhang et al., 2008). The results of the Zhang et al. (2008) study provided support for the hypothesis of a compensatory response of decreased steroid production in fish exposed to exogenous hormones. Both sexes also responded with an upregulation in *esr1* after estrogen exposure which is in agreement with what has been observed in adult fathead minnows (Filby et al., 2006).

Gene expression responses in the current study differed somewhat from what has been reported in various other studies of fish exposed to exogenous hormones. For example, we saw a downregulation of *cyp19a* expression in females exposed to EE_2 and 17β-TRB, which is opposite of the response seen in other hormone exposure studies in fish (Filby et al., 2006; Zhang et al., 2008). Johns et al. (2011) saw an upregulation of *star* with 2 ng/L EE_2 exposure to fathead minnow embryos, which is different than the downregulation of *star* observed in the present study with EE_2. These differences could be due to the use of different life stages (adults in Filby et al., 2006 and Zhang et al., 2008; pre-gametogenesis in Johns et al., 2011) and exposure duration (7d in Johns et al., 2011). Production pathways of steroid hormones likely have differing sensitivities to exogenous hormones pre-, during, and post-gametogenesis. This emphasizes the importance of investigating various life stages and exposure conditions as they can reveal different modes of action and organism sensitivity to exogenous hormone exposure. Ekman et al. (2011) saw a decrease, then an increase in *cyp19a* expression in adult female fathead minnows with continued exposure to 17β-TRB, also emphasizing the importance of exposure duration and associated effects on temporal changes in gene expression. In the current study we could possibly have seen a recovery of the negative feedback observed if we had continued to assess gene expression at a later developmental time or after a longer exposure duration.

4.2. CAFO chemical mixture

A wide range was observed in the concentrations of chemicals present in the laboratory CAFO chemical mixture, which is realistic

as large variation in chemical concentrations is commonly observed in our CAFO field sites with large spikes after rainfall events (Gall et al., 2011; Leet et al., 2012). The higher concentration of AND compared to all other hormones in the treatment tanks is likely explained by the background level of AND detected in the control tanks, which is naturally excreted by the fish. Estrogens (E_1 and 17β-E_2) were also detected in the control tanks. These findings are consistent with our data from control fish streams (Leet et al., 2012). This information is rarely measured in fish studies evaluating hormonal effects (which are usually conducted by exposing several fish of mixed genders/tank), and although hormone concentrations in the control tanks was low (7–16 ng/l for AND, and 0.2–1.3 ng/L for estrogens) their potential impact on the results observed should not be ignored.

Fish exposed for 45 days to the CAFO mixture were significantly larger and heavier compared to controls. Low concentrations of steroids have been used in aquaculture to induce growth in fish (Pickering, 1993; Woltering, 1984). Weber et al. (2013) observed an increase in larval head size in zebrafish (*Danio rerio*) exposed to low concentrations of atrazine (<30 μg/L), and Ralston-Hooper et al. (2010) observed increased growth in *Hyalella azteca* exposed to atrazine. Leet et al. (2012) also observed increased growth in feral fish in CAFO impacted ditches, as well as in fathead minnows exposed *in situ* to the ditchwater throughout development (0–45 dph). The similar response in these studies indicates there may be a common factor in the field and laboratory leading to increases in growth. Since temperature, DO, and food availability were controlled in the laboratory experiment this indicates the chemicals in the mixture (hormones, atrazine, and nitrate) may be influencing this response.

A large proportion of exposed males (84%) had ovarian cavities in their testes (compared to 0% in the control group). Concentrations of 17β-E2 and other estrogens in the present mixture study were below those known to cause feminizing effects in male fish (Table A.2). Ovarian cavities in male fish have been used as an indicator of feminization from exogenous hormone exposure during development (van Aerle et al., 2002; Brion et al., 2004). So the presence of ovarian cavities in males from our study could be due to the additive effect of estrogens in mixture. Interestingly, over the 45 days trial fish were exposed to higher levels of androgen equivalents than estrogen equivalents (Table 1). It is possible that the exogenous androgens in this mixture are being aromatized to estrogens leading to greater internal E_2 dose (Iwamatsu et al., 2006). However, if this were the case we would have expected to see a similar response in the gene expression profile as with the EE_2 exposure in the single hormone exposure study (Section 4.1), but no significant alterations in gene expression were seen. The genes analyzed here may not be sensitive enough markers to be used in mixture experiments where there are undoubtedly interactions between hormones with opposing modes of action. These particular genes did not shed clear light on the pathway impacting the phenotypic alteration observed (the presence of ovarian cavities). The "estrogenic" response observed histologically in this study demonstrates that there may not be a simple way to use additive effects to predict what will happen with mixtures of steroids with differing modes of action, as can be done for mixtures with similar modes of action (Brian et al., 2005; Silva et al., 2002).

Results from this laboratory experiment are in contrast to the masculinizing effects observed in the *in situ* exposure of fathead minnows to CAFO ditchwater over the same length of time during development (Leet et al., 2012). That study resulted in a significantly male skewed sex ratio in fish exposed to CAFO ditchwater compared to control fish reared in the laboratory. There were uncontrolled factors in the *in situ* study, such as spikes in temperature and inundation of ditch sediment into tanks, which may have contributed to the "androgenic" effect that was not observed in the current laboratory experiment (where sex ratios were not significantly different). However, differences in hormone concentrations the fish were exposed to may have played a role as well. Overall the average concentrations of hormones in the laboratory study were higher than the average concentrations in the *in situ* study (Leet et al., 2012), but there were spikes in AND and *T* (combined) in the *in situ* study that were higher than the maximum concentrations of these natural androgens in the laboratory study. It is difficult to distinguish at this time which of these factors may be leading to the differing "estrogenic" and "androgenic" effects seen in these two studies. This again indicates the complexity of alterations in fish responses to hormone mixtures, particularly hormones with differing modes of action.

5. Conclusions

In conclusion, we assessed sex-specific responses of early life stage fathead minnows to individual synthetic hormone exposures as well as to a mixture of hormones, pesticides, and nutrients similar to our CAFO field sites. Since sex identification was not previously possible in larval/juvenile fathead minnows, this is the first study to evaluate sex-specific responses of contaminant exposure in this species at this life stage. The single hormone study showed a negative feedback response to exogenous hormone exposure through downregulation of genes involved in steroid hormone production. The CAFO chemical mixture study showed no significant alterations in gene expression; however, histological data indicate a feminization of testes. The studies presented here can serve as a starting point to gain a clearer understanding of how exogenous hormones and chemical mixtures impact gonadal development in gonochoristic teleosts.

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Appendix A.

See Fig. A.1 and Tables A.1 and A.2.

Table A.1
Mean ± SE survival and total body length measured at 20 days post hatch for the single hormone experiment.

Treatment	Survival (%)	Overall body length (mm)	Male body length (mm)	Female body length (mm)
Control	97	7.7 ± 0.2	7.9 ± 0.4	7.6 ± 0.2
Estrogen	80	8.0 ± 0.1	8.0 ± 0.1	8.0 ± 0.1*
Androgen	89	7.5 ± 0.1	7.6 ± 0.2	7.4 ± 0.1*

* Significant differences from controls $p < 0.05$.

Table A.2
Summary of lowest observable effect concentrations (LOECs) for steroid hormone exposures during fish early life stages.

	Fish species	LOEC	Effect	Reference
Estrogen				
E_1	Zebrafish	14 ng/L	Induction of VTG	Holbech et al. (2006)
17β-E_2	Zebrafish	54 ng/L	Induction of VTG and skewed ratio toward females	Holbech et al. (2006)
	Japanese medaka	32 ng/L	Feminization of genotypic males based on secondary sex characteristics	Hagino et al. (2001)
	Fathead minnow	50 ng/L	Induction of VTG	Tyler et al. (1999)
	Rare minnow	25 ng/L	Induction of VTG	Liao et al. (2009)
E_3	Zebrafish	600 ng/L	Induction of VTG	Holbech et al. (2006)
Androgens				
T	Japanese medaka	100 μg/L		
17β-TRB	Zebrafish	9.5 ng/L	All male population	Holbech et al. (2006)

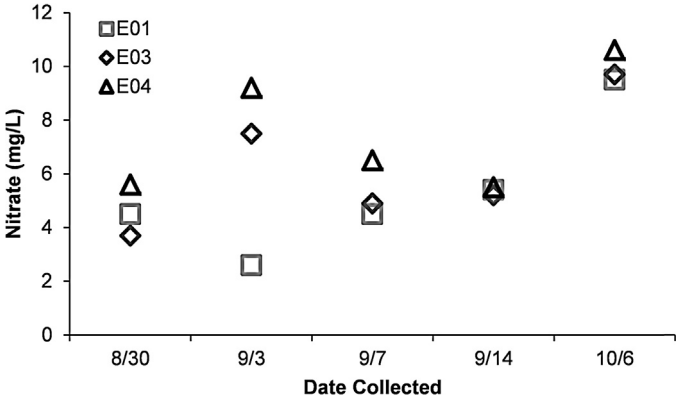

Fig. A.1. Nitrate measurements at various time points during the 45 days mixture exposure study in three different replicate tanks, E01, E03, and E04.

References

Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., et al., 2003. Effects of the androgenic growth promoter 17-beta trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ. Toxicol. Chem. 22, 1350–1360, http://dx.doi.org/10.1897/1551-5028 022<1350:EOTAGP>2.0.CO;2.

Brian, J.V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., et al., 2005. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environ. Health Perspect. 113, 721–728, http://dx.doi.org/10.1289/ehp.7598.

Brion, F., Tyler, C.R., Palazzi, X., Laillet, B., Porcher, J.M., Garric, J., Flammarion, P., 2004. Impacts of 17b-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). Aquat. Toxicol. 68, 193–217, http://dx.doi.org/10.1016/j.aquatox.2004.01.022.

Burkholder, J., Libra, B., Weyer, P., Heathcote, S., Kolpin, D., Thorne, P.S., et al., 2007. Impacts of waste from concentrated animal feeding operations on water quality. Environ. Health Perspect. 115, 308–312, http://dx.doi.org/10.1289/ehp.8839.

Cripe, G.M., Hemmer, B.L., Raimondo, S., Goodman, L.R., Kulaw, D.H., 2010. Exposure of three generations on the estuarine sheepshead minnow (*Cyprinodon variegatus*) to the androgen, 17β-trenbolone: effects on survival, development and reproduction. Environ. Toxicol. Chem. 29, 2079–2087, http://dx.doi.org/10.1002/ete.261.

Denny, J.S., Tapper, M.A., Schmieder, P.K., Hornung, M.W., Jensen, K.M., Ankley, G.T., et al., 2005. Comparison of relative binding affinities of endocrine active compounds to fathead minnow and rainbow trout estrogen receptors. Environ. Toxicol. Chem. 24, 2948–2953, http://dx.doi.org/10.1897/04-595R.1.

Ekman, D.R., Villeneuve, D.L., Teng, Q., Ralston-Hooper, K.J., Martinovic-Weigelt, D., Kahl, M.D., Jtensen, K.M., et al., 2011. Use of gene expression, biochemical and metabolite profiles to enhance exposure and effects assessment of the model androgen 17β-trenbolone in fish. Environ. Toxicol. Chem. 30, 319–329, http://dx.doi.org/10.1002/etc.406.

Filby, A.L., Thorpe, K.L., Tyler, C.R., 2006. Multiple molecular effect pathways of an environmental oestrogen in fish. J. Mol. Endocrinol. 37, 121–134, http://dx.doi.org/10.1677/jme.1.01997.

Gall, H.E., Sassman, S.A., Lee, L.S., Jafvert, C.T., 2011. Hormone discharges from a midwest tile-drained agroecosystem receiving animal wastes. Environ. Sci. Technol. 45, 8755–8764, http://dx.doi.org/10.1021/es2011435.

Iwamatsu, T., Kobayashi, H., Sagegami, R., Shuo, T., 2006. Testosterone content of developing eggs and sex reversal in the medaka (*Oryzias latipes*). Gen. Comp. Endocrinol. 145, 67–74, http://dx.doi.org/10.1016/j.ygcen.2005.07.003.

Jensen, K.M., Makynen, E.A., Kahl, M.D., Ankley, G.T., 2006. Effects of the feedlot contaminant 17α-trenbolone on reproductive endocrinology of the fathead minnow. Environ. Sci. Technol. 40, 3112–3117, http://dx.doi.org/10.1021/es052174s.

Johns, S.M., Denslow, N.D., Kane, M.D., Watanabe, K.H., Orlando, E.F., Sepúlveda, M.S., 2011. Effects of estrogens and antiestrogens on gene expression of fathead minnow (*Pimephales promelas*) early life stages. Environ. Toxicol. 26, 195–206, http://dx.doi.org/10.1002/tox.20545.

Leet, J.K., Sassman, S.A., Meyer, J.L., Goforth, R.R., Gall, H.E., Javfert, C.T., et al., 2012. Assessing impacts of land-applied manure from concentrated animal feeding operations on fish populations and communities. Environ. Sci. Technol. 46, 13440–13447, http://dx.doi.org/10.1002/tox.20545.

Leet, J.K., Lesteberg, K.E., Schoenfuss, H.L., Olmstead, A.W., Amberg, J.J., Ankley, G.T., et al., 2013. Sex-specific gonadal and gene expression changes throughout development in fathead minnow. Sex Dev. 7, 303–337, http://dx.doi.org/10.1159/000353877.

Olmstead, A.W., Villeneuve, D.L., Ankley, G.T., Cavallin, J.E., Lindberg-Livingston, A., Wehmas, L.C., et al., 2011. A method for the determination of genetic sex in the fathead minnow *Pimephales promelas*, to support testing of endocrine-active chemicals. Environ. Sci. Technol. 45, 3090–3095, http://dx.doi.org/10.1021/es103327r.

Orberdorster, E., Cheek, A.O., 2000. Gender benders at the beach: endocrine disruption in marine and estuarine organisms. Environ. Toxicol. Chem. 20, 23–26, http://dx.doi.org/10.1897/1551-5028(2001)020<0023:GBATBE>2.0.CO;2.

Pickering, A.D., 1993. Growth and stress in fish production. Aquaculture 111, 51–63, http://dx.doi.org/10.1016/0044-8486(93)90024-S.

Ralston-Hooper, K.J., Hardy, J., Hahn, L., Ochoa-Acuña, H., Lee, L.S., Mollenhauer, R., Sepúlveda, M.S., 2009. Acute and chronic toxicity of atrazine and its metabolites deethylatrazine and deisopropylatrazine on aquatic organisms. Ecotoxicology 18, 899–905.

Ralston-Hooper, K.J., Adamec, J., Jannash, A., Mollenhauer, R., Ochoa-Acuña, H., Sepúlveda, M.S., 2010. Use of GC × GC/TOF–MS and LC/TOF–MS for metabolomic analysis of *Hyalella azteca* chronically exposed to atrazine and its primary metabolite, desethylatrazine. J. Appl. Toxicol. 31, 399–410, http://dx.doi.org/10.1007/s10646-009-0351-0.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative Ct method. Nat. Protoc. 3, 1101–1108, http://dx.doi.org/10.1038/nprot.2008.73.

Sharpe, R.L., Woodhouse, A., Moon, T.W., Trudeau, V.L., MacLatchy, D.L., 2007. β-Sitosterol and 17β-estradiol alter gonadal steroidogenic acute regulatory protein (StAR) expression in goldfish *Carassius auratus*. Gen. Comp. Endocrinol. 151, 34–41, http://dx.doi.org/10.1016/j.ygcen.2006.11.005.

Shved, N., Berishvili, G., D'Cotta, H., Baroiller, J.F., Segner, H., Reinecke, M., 2008. Environmentally relevant concentrations of 17α-ethinylestradiol (EE2) interfere with the growth hormone (GH)/Insulin-like growth factor (IGF)-I system in developing bony fish. Toxicol. Sci. 106, 93–102, http://dx.doi.org/10.1093/toxsci/kfn150.

Silva, E., Rajapakse, N., Kortenkamp, A., 2002. Something from "nothing" – eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ. Sci. Technol. 36, 1751–1756, http://dx.doi.org/10.1021/es0101227.

Stoeckel, J.A., Morris, J., Ames, E., Glover, D.C., Vanni, M.J., Renwick, W., et al., 2012. Exposure times to the spring atrazine flush along a stream-reservoir system. J. Am. Water Resour. Assoc. 48, 616–634, http://dx.doi.org/10.1111/j.1752-1688.2011.00633.x.

Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P., et al., 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ. Sci. Technol. 37, 1142–1149, http://dx.doi.org/10.1021/es0201348.

Tyler, C.R., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28, 319–361, http://dx.doi.org/10.1080/10408449891344236.

Uguz, C., 2008. Histological evaluation of gonadal differentiation in fathead minnow (*Pimephales promelas*). Tissue Cell 40, 299–306, http://dx.doi.org/10.1016/j.tice.2008.02.003.

United States Environmental Protection Agency, 2006. Histopathology Guidelines for the Fathead Minnow (*Pimephales promelas*) 21-Day Reproduction Assay. USEPA, Duluth, MN.

van Aerle, R., Pounds, N., Hutchinson, T.H., Maddix, S., Tyler, C.R., 2002. Window of sensitivity for the estrogenic effects of ethinylestradiol in early

lifestages of fathead minnow *Pimephales promelas*. Ecotoxicology 11, 423–434, http://dx.doi.org/10.1023/A:1021053217513.

Weber, G.J., Sepúlveda, M.S., Peterson, S.M., Lewis, S.S., Freeman, J.L., 2013. Transcriptome alterations following developmental Atrazine exposure in zebrafish are associated with disruption of neuroendocrine and reproductive system function, cell cycle, and carcinogenesis. Toxicol. Sci. 132, 458–466, http://dx.doi.org/10.1093/toxsci/kft017.

Wilson, V.S., Cardon, M.C., Gray Jr., L.E., Hartig, P.C., 2007. Competitive binding comparison of endocrine-disrupting compounds to recombinant androgen receptor from fathead minnow, rainbow trout, and human. Environ. Toxicol. Chem. 26, 1793–1802, http://dx.doi.org/10.1897/06-593R.1.

Woltering, D.M., 1984. The growth response in fish chronic and early life stage toxicity tests: a critical review. Aquat. Toxicol. 5, 1–21, http://dx.doi.org/10.1016/0166-445X(84)90028-6.

Zhang, H., Kong, F.X., Wang, S.H., Yu, Y., Zhang, M., 2009. Vitellogenin induction by a mixture of steroidal estrogens in freshwater fishes and relevant risk assessment. Environ. Toxicol. 24, 484–491, http://dx.doi.org/10.1002/tox.20453.

Zhang, X., Hecker, M., Park, J., Tompsett, A.R., Newsted, J., Nakayama, K., et al., 2008. Real-time PCR array to study effects of chemicals on the hypothalamic–pituitary–gonadal axis of the Japanese medaka. Aquat. Toxicol. 88, 173–182, http://dx.doi.org/10.1016/j.aquatox.2008.04.009.